Review Article

Stem Cell INVESTIGATION

Oncolytic virus as a cancer stem cell killer: progress and challenges

Jingzhen Ding

Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093, USA
Correspondence to: Jingzhen Ding. Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093, USA. Email: dingjz8421@gmail.com.

Abstract: Oncolytic viruses (OVs), which were discovered more than one century ago, have been used in multiple clinical trials for cancer therapy. OVs specifically target cancer cells when sparing normal cells by exploiting biochemical differences between normal and tumor cells. Hence oncolytic virotherapy is more specific at targeting cancer cells compared with conventional anti-cancer therapy. Apart from the lack of specificity, conventional anti-cancer therapies also often witness relapse and incomplete cure of cancer. One hypothesis explaining this phenomenon is that a subpopulation of cancer cells, known as cancer stem cells (CSCs), are resistant to conventional therapies, possibly due to its self-renewal and differentiation abilities. With the discovery of CSCs, researchers have been trying to explain whether OVs are well suited to eliminate CSCs. Two explanations for postulating OVs as ideal candidates for cancer therapy have been proposed: first, OVs are not subject to the same mechanisms responsible for chemotherapy and radiation resistance; second, viruses could be harnessed to express therapeutic transgenes that specifically target the features unique to CSCs or the properties CSCs rely on for self-renewal and differentiation. Indeed, initial studies suggest that OVs could effectively target CSCs in multiple tumor types. The focus of this review is to highlight recent studies related to the application of OVs on targeting CSCs, based on which, the challenges and perspectives for further research in this field will also be discussed.

Keywords: Cancer stem cells (CSCs); oncolytic viruses (OVs); cancer therapy

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Introduction to cancer stem cells (CSCs)

CSCs were not isolated until the mid-1990s, but the concept was proposed by two German pathologists, Rudolph Virchow and Julius Cohnheim, more than one and a half centuries ago. The two scientists both noted histological similarities between developing fetuses and certain cancers (1), and then propose that cancer cells might be originated from embryonic tissue. Due to the limited development of technology back then, a formal proof of this conceptualization had not arisen until 1963 when Bruce *et al*. observed that only a very small portion of lymphoma cells could form *in vitro* colonies and initiate tumorigenesis in a xenograft transplant (2). A few decades later, in 1994, Dick and colleagues demonstrated that human acute myeloid leukemia (AML) could be regenerated after transplantation of leukemic stem cells possessing $CD34^+CD38^-$ cell surface markers in non-obese diabetic mice (3). Although the occurrence of CSCs was rare (~$1/10^6$), a single leukemic stem cell could give rise to the repertoire of populations of leukemia cells (4). Since then, CSCs have been isolated from a number of tumor types, including brain, breast, colon, ovarian, head and neck, pancreas and liver (5-13). According to the CSC hypothesis, only a small subpopulation of cells is tumorigenic: that is, only CSCs with the ability of self-renewal and self-differentiation can give rise to the variety of rapidly proliferating and differentiated cells that make up the bulk of a tumor.

Till now, the most common way to identify and

isolate CSCs is to use the phenotypic markers, based on the expression of which, flow cytometry and magnetic-activated cell separation are widely used to enrich CSCs (14,15). Additionally, functional assays, such as serial transplantation in animal models, have also been applied complementarily to define CSCs. Typically, cells are isolated from existing tumors by using surface markers or enzymatic activity, and then tested for their ability to form tumor spheroids *in vitro* or tumors in secondary recipients *in vivo* when orthotopically xenografted into immunodeficiency mice (14).

Despite the progress of CSC research, CSC hypothesis is still under debates due to the following two major concerns. First, many CSC markers identified are demonstrated not exclusively limited to CSC populations, but also to healthy tissue (16), which casts doubt on the reliability of using surface biomarkers or enzymatic activity to identify CSCs. Besides, recent discovery also proposed a dynamic state of marker expression on CSCs, that is, CSC populations may only transiently express the CSC markers (17). Therefore, in order to further elucidate the cellular biology of CSCs, better understanding is required of both the specificity and stability of the CSC markers.

The second concern was raised by a study by Kelly *et al*. group. They demonstrated that when lymphomas and leukemias of mouse origin are transplanted into histocompatible mice, a very high frequency of tumor cells (1 in 10) could seed new tumor growth (18). Obviously, their findings challenge the idea that the tumor growth is driven by a rare subpopulation of tumor cells, indicating the limitations of human tumor cells' ability to grow in a foreign (mouse) microenvironment.

Regardless of the uncertainty of the specificity and stability of CSC markers, as well as the debates on the frequency of CSC populations, CSC hypothesis is under dynamic refined as research progresses. With growing insight into the mechanism of CSC development, function, and interplay with the tumor microenvironment, CSCs hold great promise as a therapeutic target for reducing relapse rates and improving long-term outcome for patients with many types of cancers.

CSCs *vs.* normal stem cells

The term "CSCs" was originally coined according to its similarity to bona fide normal stem cells (19). Like normal stem cells, CSCs also have the ability to self-renew and self-differentiate, by which, they are able to produce daughter cells that constitute the bulk of a tumor similar to the way normal stem cells generate an organ. In particular, CSCs express similar markers to normal stem cells in the same tissues (19-21), such as CD34 in both leukemia CSCs and normal hematopoietic stem cells (3,22), as well as CD133 in brain cancer and normal neural stem cells (NSC) (23-26). Besides, both CSCs and normal stem cells share similar pathways for their proliferation and self-renewal, such as Hedgehog, Wnt and Notch pathways (27-30).

Another feature CSCs share with normal stem cells is a specialized microenvironment, named niche. In 1978, Schofield first proposed the existence of a niche for hematopoietic stem cells that serves as the key regulator of the balance between self-renewal and differentiation (31). The regulation of the niche occurs through cell-to-cell interactions, the extracellular matrix, and the balance of signals received from embryonic signaling pathways (32-34). For example, the NSCs lie within a vascular niche, in which endothelial cells secrete factors that promote stem cell survival and self-renewal and are thought to be a key component of the NSC niche (35-38). Similarly, endothelial cells interact selectively with $Nestin^+/CD133^+$ brain cancer cells that include the CSC fraction. Increasing the number of endothelial cells expanded the numbers of self-renewing $Nestin^+/CD133^+$ cancer cells and accelerated the initiation and growth of tumors, and vice versa (39).

The term cancer "stem cell" harbors the hypothesis that cells in the tumorigenic fraction harbor all properties of normal stem cells. However, whether this is true is the subject of much debate. One difference between normal stem cells and CSCs might be their relationship with niches. Stem cells exist within protective niches composed of a number of differentiated cell types that provide direct cell contacts and secreted factors to maintain stem cells primarily in a quiescent state (40,41). However, it was proposed that CSCs might escape from the niche control with either intrinsic mutations or extrinsic aberrant niche microenvironment (42,43). If it is true that CSCs depend upon aberrant niches, then these niches might represent targets for treatments of cancer.

In 2006, Yilmaz *et al*. group reported a solid piece of evidence showing the difference between CSCs and normal stem cells. They find that Pten deletion causes the generation of transplantable leukemia CSCs, but results in the depletion of normal hematopoietic stem cells (44). Their findings provide a potential therapeutic target pathway that has distinct effects on normal stem cells and

CSCs within the same tissue.

CSCs are resistant to conventional cancer therapies

The mechanisms by which CSCs are resistant to conventional therapies are very diverse, including slow cell cycle kinetics, efficient DNA repair, upregulation of anti-apoptotic proteins and multidrug resistance type membrane transporters (13,16,45-47). All these evasion mechanisms enable CSCs to repopulate the tumor mass, leading to recurrence of a cancer.

Interestingly, most of the known resistance mechanisms of CSCs to conventional therapies are involved in the stem-like properties embodied by CSCs, such as relative quiescence and high-level of drug efflux transporters. The quiescence allows CSCs escape drugs that are designed only to highly proliferative cells (other cancer cells), such as DNA synthesis inhibitors (16,48); and the upregulation of membrane transporters, such as ATP-binding cassette protein efflux pump ABCG2, makes them drug resistant (20,21). Therefore, the similarity between CSCs and normal stem cells confound an already difficult situation of cancer therapies, that is, besides the concern with the differentiation of CSCs from other tumor cells and normal cells, what is in also great need is a deeper delving into the unique properties of CSCs different from normal stem cells.

Introduction to oncolytic viruses (OVs)

The historical record of OV therapy began in 1912, when DePace reported tumor regression after inoculation of an attenuated rabies vaccine (49), inspired by which, Levaditi and Nicolau, reported viral oncolysis in the mouse in 1922 (50). A human trial was not documented until 1950, when Pack used an attenuated rabies virus against melanoma and yielded a remarkable partial remission (51). After that, numerous emerging OVs were used to treat patients suffering from various types of cancer (52-55). Such viruses fall into three broad categories: (I) wild-type viruses that do not typically infect human cells but are cytotoxic to human cancer cells; (II) attenuated viruses, either by mutations or serial passage, specifically targeting cancer cells; (III) larger viruses that can be armed to express transgenes (16,56).

OVs target cancer cells in four ways: (I) OVs kill tumor cells that contain altered signaling pathways different from normal cells; (II) OVs attack tumor cells due to either genetically engineered mutations or harnessed foreign genes that directly or indirectly result in cancer cell death; (III) OVs infection of cancer cells induces antitumor immunity (57-59); (IV) In relatively rare cases, viruses produce cytotoxic protein during replication in cells, with the protein itself then causing damage to the cell. For example, adenoviruses express the E3 11.6-kDa death protein that is cytotoxic to cells (60).

Targeting CSCs with OVs

OVs kill cancer cells in a way that differs from traditional therapies and thus are able to elude the typical mechanisms in which CSCs are used to resist current chemotherapy and radiotherapy. So far, various OVs and mutant strains have been reported to successfully target different types of CSCs. The list could be found in two review articles (16,57). Here, I will focus on the application of harnessed OVs, and the combination use of OVs and conventional cancer therapies.

Besides initial screening of available OVs for their potentials of killing CSCs, genetic manipulation of OVs has been used to enhance either the efficiency or the specificity. Basically, there are four strategies to be considered. First, engineered OVs are designed to have increased tumor penetration. For example, Zhu *et al.* used a herpes simplex virus (HSV) carrying endostatin-angiostatin (VAE) to target glioblastoma-derived CSCs (61). VAE specifically inhibits the secretion of vascular endothelial cells and fibroblasts growth factors and consequently disrupts the vascular niche, therefore exposing GSCs for the virus attack. Similarly, a chondroitinase adenosine triphosphate-binding cassette removes the chondroitin sulfate from the tumor extracellular matrix proteoglycans, which also helps HSV spread throughout the glioma spheroids more efficiently and thus enhances anti-tumor activity *in vivo* (62).

Second, given that cancer cell antigen is a weak antigen for host immune sensitization, OVs armed with antitumor immunity factors show promises as well. An HSV armed with talimogene laherparepvec, a cytokine granulocyte macrophage colony stimulating factor, showed promises on metastatic melanoma (63). Another HSV, when armed with murine IL-12, lysed human glioblastoma ($CD133^+$) CSC-like cells (GSCs) and targeted mouse GSC *in vivo* (64).

Third, as virus replication is optimal in highly proliferating cells, the ability to induce programmed cell

death is likely to increase the therapeutic potential of the OVs for CSCs, which are largely quiescent cells. For example, a multimutated HSV synergized with the PI3K/Akt pathway inhibitors in killing CSCs through enhanced apoptosis (65).

Lastly, $CD133^+$-targeted measles viruses enhanced oncolytic activity of $CD133^+$ specific viruses when compared with the unmodified ones (66).

As for the combination of OVs with chemo- or radiation therapy, it will likely to achieve superior outcome of eliminating CSCs. OVs may sensitize cells to radiation, and radiation can enhance viral infection, replication, and gene expression, resulting in greater tumor cell death (67). Low-dose chemotherapy with agents like cyclophosphamide can reduce the antiviral immune response and thus enhance oncolysis (68). Besides, virotherapy may complement high-dose chemotherapy that causes toxic side effect. For example, the combination of low-dose etoposide with G47Δ significantly kills GSC-derived tumors (69), and so does the combination of alkylating agent temozolomide (TMZ) and G47Δ in killing GSCs (70).

Challenges and perspectives

The designs of conventional therapies are usually based on tumor subtypes, stage, grade and other molecular files. However, due to the tumor heterogeneity, both within the tumor and between patients, the efficacy of current treatment strategies is limited. In addition, chemo and radio-resistance and subsequent patient relapse are still a big concern. By contrast, OVs kill cancer cells, including CSCs in a way that is irrespective of tumor subtypes, and also with minimal toxic side effects to their normal cell and tissue counterparts. Apparently, OVs provide a lot of excitement about developing therapies that target CSCs, but great amount of efforts still need to be put in toward a more successful clinic translation.

To begin with, a better understanding of CSC biology is required for the development of OV therapy that specifically targets CSCs. It requires a more accurate differentiation of CSCs from other tumor cells, normal cells and particularly normal stem cells so that OV may specifically target CSCs. The identification of CSCs includes unique surface antigens, the niche and signaling pathways. Besides, it is equally important to investigate the origins of CSCs and the occurring frequency of CSCs in a broad range of human cancers. It is possible that the number of CSCs may directly correlate with the stage of tumor development. In that case, the occurrence and abundance of CSCs in primary and metastatic, especially in benign tumors should be different. In addition to the intrinsic driving forces, an extrinsic stimulation, such as chemotherapy, may also regulate the numbers of CSCs, or the biological features of CSCs. Addressing all these questions will present a multifaceted landscape of CSCs.

Another way to improve the efficiency of OV includes enhancing virus delivery, replication and increasing the tumor-directed immune response. In addition, the combination therapy with chemotherapeutics, radiation, monoclonal antibodies, and small-molecule inhibitors may also achieve better outcomes. But currently, except some armed viruses designed specifically for targeting CSCs, most studies use viruses that generally kill both cancer cells and CSCs. Of course, with better understanding of CSCs, OVs could be harnessed to meet the specificity, but discovery of new OVs is also important. Further, although physical and physiological barriers within the tumor microenvironment limit viral spread to CSCs, it is possible to turn these barriers into incubators, that is, by using the unique features of CSC niche, a OV can be designed to replicate only when it reaches the CSC niche.

The last but not the least challenge for the researchers is to discover the unique mechanisms of OVs killing CSCs. Most studies have been focusing on the phenotypic features of OVs on CSCs, and only a few have reported the underlying mechanisms, such as induction of autophagy and robust of DNA damages (70,71). However, these observations on mechanisms are not exclusive to CSCs, which limits the significance.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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